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YidC-mediated Membrane Insertion of Assembly Mutants of Subunit c of the F_1F_0 ATPase*

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YidC is a member of the OxaI family of membrane proteins that has been implicated in the membrane insertion of inner membrane proteins in *Escherichia coli*. We have recently demonstrated that proteoliposomes containing only YidC support both the stable membrane insertion and the oligomerization of the c subunit of the F_1F_0 ATP synthase (F_0c). Here we have shown that two mutants of F_0c unable to form a functional F_1F_0 ATPase interact with YidC, require YidC for membrane insertion, but fail to oligomerize. These data show that oligomerization is not essential for the stable YidC-dependent membrane insertion of F_0c consistent with a function of YidC as a membrane protein insertase.

Membrane protein targeting and insertion in *Escherichia coli* generally occurs via the highly conserved SRP/SecYEG pathway (1, 2). In addition, another membrane protein, YidC, that associates with SecYEG has been implicated in membrane protein insertion (3). YidC is a member of the Alb3/OxaI/YidC family of membrane proteins that function in promoting membrane insertion in chloroplasts, mitochondria, and bacteria (4, 5). YidC has been implicated in various steps of membrane protein insertion and assembly. YidC was shown to cross-link to the transmembrane regions of nascent membrane proteins (3, 6, 7) and was therefore proposed to function in concert with the Sec translocase to transfer transmembrane regions of Sec-dependent proteins into the lipid bilayer. Indeed, recent evidence shows that YidC is essential for the Sec translocase-mediated membrane insertion of subunit a of the cytochrome bo_3 oxidase subunit (8–10). YidC has also been implicated in the folding of LacY but appears not to be essential for its membrane insertion (11). Importantly, YidC also functions independently of the Sec translocase in membrane protein insertion. This was first demonstrated for small phage proteins such as M13 procoat (12)

and Pf3 coat (13) that were previously suggested to insert “spontaneously” into the lipid bilayer (14). The c subunit of the F_1F_0 ATP synthase (F_0c) was shown to be an authentic substrate for this YidC-only pathway (15, 16). F_0c is a small inner membrane protein with two transmembrane segments that assembles into a decameric ring structure in *E. coli* (17). The c-ring is associated with two b subunits and one a subunit (F_0b and F_0a , respectively) and forms the membrane-embedded F_0 sector. The catalytic F_1 domain is peripherally bound to the F_0 sector at the cytosolic side of the membrane. The F_1F_0 ATP synthase converts the energy stored in a transmembrane electrochemical proton gradient into ATP and plays a central role in the energy metabolism of the cell. ATP synthesis or hydrolysis occurs on three catalytic sites in the F_1 sector, whereas H^+ transport occurs through the F_0 subcomplex at the interface between F_0a and the F_0c ring (18–20).

Using an *in vitro* system it was demonstrated that YidC is required and sufficient for the stable membrane insertion of F_0c in its correct topology (15). Membrane-inserted F_0c was found to assemble into a large oligomeric complex with a size reminiscent of that of the F_0c ring observed *in vivo*. Interestingly, all known proteins that strictly require YidC for membrane insertion, the major coat proteins of the bacteriophages Pf3 and M13 (21), F_0c of the F_1F_0 ATP synthase, and subunit a of the cytochrome bo_3 oxidase subunit, assemble into large oligomeric structures, which may point to a role of YidC in the assembly of macromolecular protein complexes. Furthermore, this raises the question of whether insertion and oligomerization are concerted processes and whether these both require the function of YidC. YidC may act solely as a membrane protein insertase mediating the membrane integration of F_0c monomers. Alternatively, YidC may function as a membrane chaperone that stabilizes the “spontaneously” membrane-inserted monomeric form of F_0c in its correct conformation to facilitate its oligomerization. Another possibility is that YidC catalyzes both the insertion and assembly reactions.

To distinguish between the putative insertase and chaperone functions of YidC we have analyzed the YidC dependence of the membrane insertion of two F_0c mutants, *i.e.* G23D and L31F, that do not support the formation of an active F_1F_0 ATP synthase (22, 23). These mutants have been suggested to be defective in assembly of the F_0 sector, but the exact nature of the assembly defect has not been elucidated (24). Here we have shown that the two F_0c mutants still insert *in vitro* into the membrane in a YidC-dependent manner but that they are unable to assemble into an oligomer. This shows that the oligomerization into the c-ring *per se* is not essential for the stable

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YidC-dependent membrane insertion of F₀C consistent with a function of YidC as a membrane protein insertase.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The *uncE114* mutant strain MM944I^q (25) was used in the *in vivo* complementation experiments and ATPase activity and Δ pH measurements. pET20AtpE was used for the *in vitro* transcription of wild-type F₀C (15). pET20AtpE-G23D and pET20AtpE-L31F were constructed by replacing the GGT codon at position 23 with GAT or the CTC codon at position 31 with TTC in the *atpE* gene, respectively. For *in vivo* expression, the NdeI/EcoRI sites surrounding *atpE* were replaced by NcoI/BamHI sites, and the resulting fragment was cloned into the plasmid pTrc99a, yielding pTrc99AtpE and its derivatives pTrc99AtpE-G23D and pTrc99AtpE-L31F.

In Vivo Complementation—The pTrc99a-based plasmids were used to transform strain MM944I^q. Cells were streaked on M63 minimal medium plates containing 0.6% succinate or 0.2% glucose with the appropriate supplements and 25 μ g/ml kanamycin and 100 μ g/ml ampicillin (25). Cells were grown for 20 h at 37 °C.

ATPase and Δ pH Measurements—Strain MM944I^q transformed with the appropriate plasmids was grown on M63 medium supplemented with 0.6% glucose, the appropriate supplements, 25 μ g/ml kanamycin, 100 μ g/ml ampicillin, and 400 μ M isopropyl-1-thio- β -D-galactopyranoside for induction until an OD₆₆₀ of 1.0. Cells were collected by centrifugation, and inner membrane vesicles (IMVs)⁵ were isolated by French pressure treatment (26). The ATPase activity of isolated IMVs at 37 °C was measured by the malachite green assay as described (27). To inhibit the activity of coupled F₁F₀ complexes 250 μ M dicyclohexylcarbodiimide was used. The Δ pH in IMVs was measured by means of the fluorescent dye 9-amino-6-chloro-2-methoxyacridine (ACMA). Reaction mixtures contained 1.25 μ g/ml IMVs and 1 μ M ACMA in 25 mM HEPES-KOH, pH 8.0, 25 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin. Reactions were started by the addition of 1 mM ATP or 1.25 mM NADH, and the ACMA fluorescence was monitored in a PerkinElmer LS50B luminescence spectrophotometer at excitation and emission wavelengths of 409 and 474 nm, respectively.

In Vitro Insertion Reaction Assays—The RiboMax transcription kit (Promega) was used for the synthesis of mRNA in a coupled transcription/translation system. Reactions were carried out for 20 min at 37 °C in the presence of proteoliposomes as described previously (28). A small sample of the reaction mixture was used as a synthesis control, and the remainder was treated with 0.4 mg/ml proteinase K for 30 min on ice in the presence or absence of 1% Triton X-100. Samples were trichloroacetic acid precipitated and analyzed by 18% SDS-PAGE and phosphorimaging.

Blue Native PAGE Analysis—F₀C was synthesized radioactively in the presence of proteoliposomes containing YidC or without protein present. After re-isolation of the F₀C-loaded

⁵ The abbreviations used are: IMV, inner membrane vesicle; ACMA, 9-amino-6-chloro-2-methoxyacridine; DDM, dodecyl maltoside.

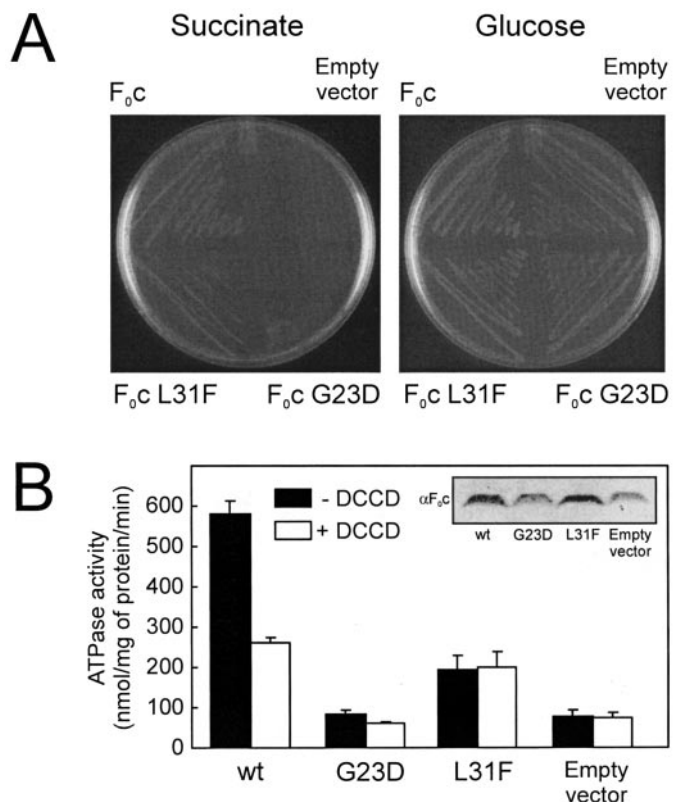


FIGURE 1. The F₀C G23D and L31F mutants are unable to form a functional F₁F₀ synthase. A, growth of *E. coli* strain MM944I^q on succinate (left panel) and glucose (right panel) minimal medium upon the expression of wild-type F₀C, F₀C G23D, F₀C L31F, or the empty plasmid. B, ATPase activity of IMVs derived from MM944I^q cells grown on 0.6% glucose expressing wild-type F₀C, F₀C G23D, or F₀C L31F. Where indicated, 250 μ M dicyclohexylcarbodiimide was used to inhibit the activity of coupled F₁F₀ complexes. Insert, IMVs were analyzed by SDS-PAGE and immunostaining using antibodies against F₀C.

(proteo)liposomes, pellets were resuspended in 25 μ l of solubilization buffer (50 mM HEPES-KOH, pH 8.0, 50 mM KCl, 10% glycerol, and 0.05% dodecyl maltoside) and incubated for 15 min on ice. Samples were then mixed with gel-loading buffer. Blue Native PAGE was performed on an 8–18% gradient gel as previously described (29).

Association of *in Vitro*-synthesized F₀C with YidC—F₀C was synthesized radioactively in the presence of YidC proteoliposomes, empty liposomes, or no membranes present. After synthesis, 0.1% DDM-solubilized YidC proteoliposomes were added to the reactions without membranes present. All reactions were subsequently treated with 0.1% DDM, and after 15 min on ice nonsolubilized proteins were removed by centrifugation through a glycerol cushion consisting of 10 mM HEPES-KOH, pH 7.5, 0.1% DDM, 20% glycerol. The supernatant was mixed with Ni²⁺-NTA-agarose beads (Qiagen, Hilden, Germany) equilibrated with buffer A (10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 20% glycerol, 0.1% DDM) containing 10 mM imidazole. The suspension was gently shaken for 2 h at 4 °C. Beads were washed with 10 volumes of buffer A containing 60 mM imidazole, and bound proteins were eluted with buffer A containing 250 mM imidazole, transferred to gel-loading buffer, and analyzed by 18% SDS-PAGE and phosphorimaging.

Other Methods—YidC was purified and reconstituted into *E. coli* phospholipids (Avanti Polar Lipids, Inc., Alabaster, AL)

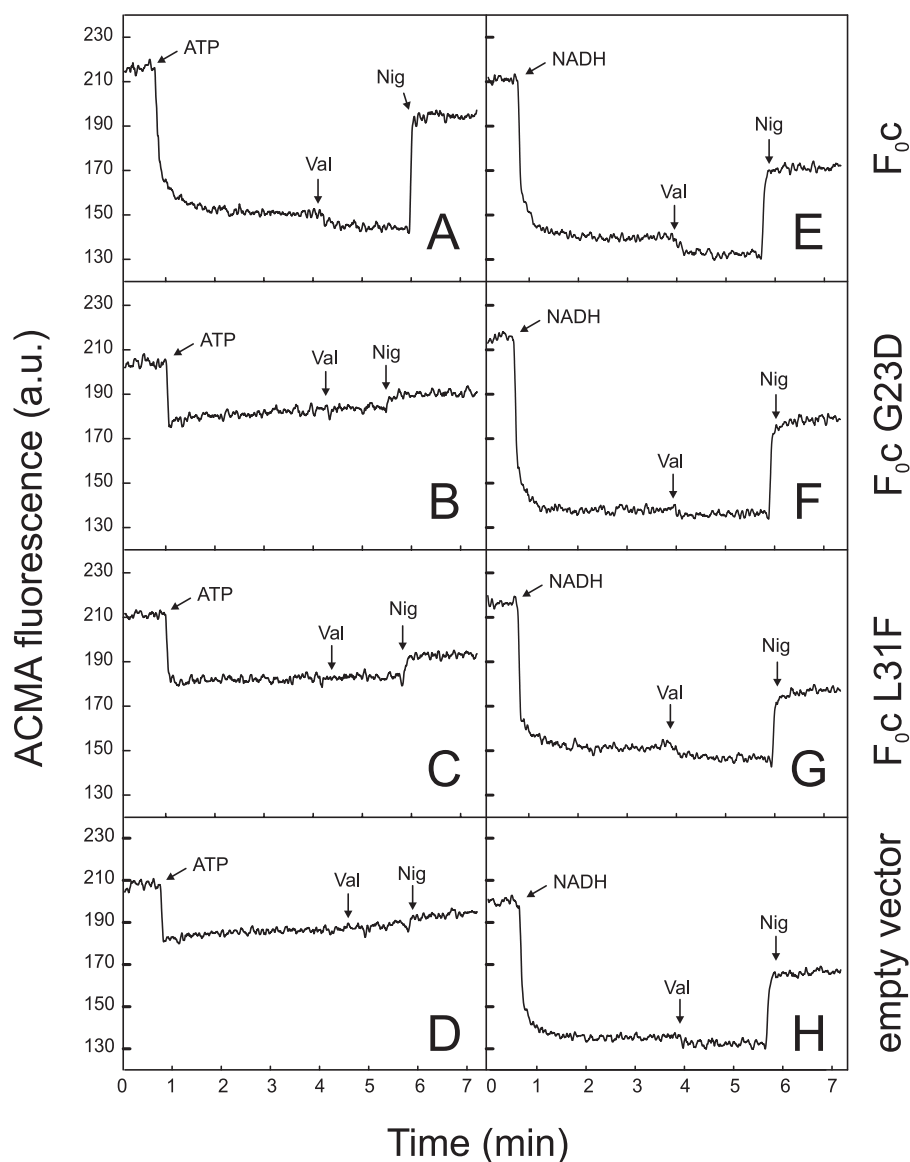


FIGURE 2. *F₀c* G23D and *F₀c* L31F are defective in proton motive force generation. MM944I^q IMVs containing wild-type *F₀c* (A, E), *F₀c* G23D (B, F), *F₀c* L31F (C, G), or no *F₀c* (D, H) were analyzed for the generation of a $\Delta\psi$ as monitored by the fluorescence quenching of ACMA. Where indicated 1 mM ATP (A–D) or 1.25 mM NADH (E–H) was added to generate a proton motive force. Valinomycin (1 μ M) was used to convert the $\Delta\psi$ into a Δ pH, while nigericin (1 μ M) was used to collapse the Δ pH.

as described (30). YidC polyclonal antiserum was raised in rabbit against purified hisYidC by Agrisera (Umeå, Sweden). *F₀c* monoclonal antiserum was a generous gift from G. Deckers-Hebestreit (University of Osnabrück, Germany).

RESULTS

***F₀c* G23D and L31F Mutants Are Defective in the Formation of an Active *F₁F₀* ATP Synthase**—Previously, two *F₀c* mutants (G23D and L31F) (22, 23) have been isolated that do not support the assembly of a functional *F₁F₀* ATP synthase, possibly as a result of a defect in the formation of a functional *F₀* sector. As both mutants have not been analyzed biochemically, we first validated their phenotype *in vivo*. For this purpose, the *atpE* (G23D) and *atpE* (L31F) mutant genes were cloned behind the inducible *trc* promoter and the resulting plasmids were trans-

formed into strain MM944I^q. This strain contains a chromosomal mutation in the *atpE* gene encoding subunit c (Q42E) that disturbs the binding of *F₁* to the *F₀* subcomplex (25). Strain MM944I^q is unable to grow on minimal medium with succinate as an energy and carbon source, whereas growth on glucose is undisturbed. Introduction of a plasmid containing wild-type *atpE* into MM944I^q fully restored growth on succinate minimal medium plates (Fig. 1A, right panel), whereas an empty plasmid or a plasmid containing *atpE* (G23D) had no effect. A plasmid containing *atpE* (L31F) also restored growth of strain MM944I^q on succinate minimal medium plates, but after overnight growth at 37 °C observed colonies were approximately half the size of the colonies with the wild-type *atpE* plasmid (Fig. 1A, left panel). Immunoblotting showed that the levels of *F₀c* in IMVs derived from cells expressing wild-type *F₀c* and *F₀c* L31F are elevated as compared with background levels of the chromosomal *atpE* gene (Fig. 1B, insert), whereas with *F₀c* G23D IMVs the level of *F₀c* is only marginally increased.

To obtain quantitative information on the activity of the *F₀c* mutants, IMVs from strain MM944I^q were analyzed for their ATPase activity and ability to generate a proton motive force. In contrast to the wild-type, both *F₀c* mutants supported only low levels of ATPase activity. When dicyclohexylcarbodiimide, an inhibitor of

proton translocation by *F₁F₀* ATP synthase, was added, the ATPase activity of the wild-type was strongly reduced whereas the activity of the mutant complexes was unaffected (Fig. 1B). To examine the effect of the *F₀c* mutants on the ability of the *F₁F₀* ATP synthase to generate a proton motive force, IMVs were incubated with either ATP or NADH and the generation of a transmembrane proton gradient (Δ pH) was monitored with the fluorescent dye ACMA. The ionophore valinomycin that converts the transmembrane electrical potential ($\Delta\psi$) into an additional Δ pH was added to ensure that the Δ pH is the only component of the proton motive force, while the ionophore nigericin was added to collapse the Δ pH. In contrast to wild-type *F₀c* (Fig. 2A), the *F₀c* G23D and *F₀c* L31F mutants were defective in the ATP-dependent generation of a Δ pH (Fig. 2, B and C). The inhibitory effect of the ionophore nigericin is some-

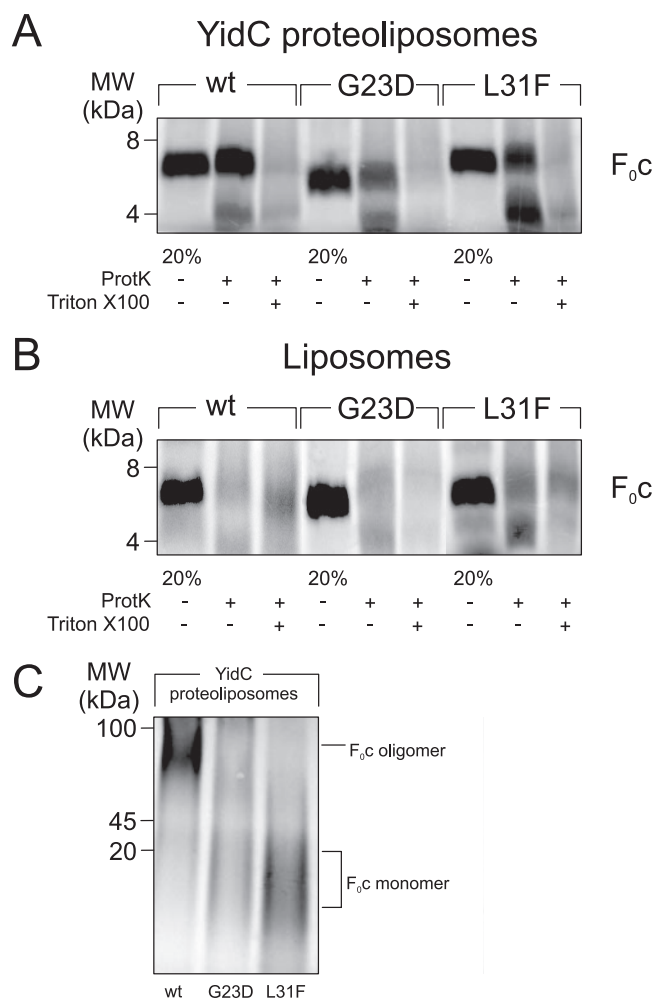


FIGURE 3. YidC-dependent insertion of F₀c G23D and F₀c L31F into proteoliposomes. F₀c was synthesized in the presence of YidC proteoliposomes (A) or liposomes (B). After translation, samples were treated with 0.4 mg/ml proteinase K (panels A and B, lanes 2, 3, 5, 6, 8, 9) in the presence (lanes 3, 6, 9) or absence (lanes 2, 5, 8) of 1% (v/v) Triton X-100. Where indicated, 20% standards of the translation reactions are shown. C, membrane-inserted F₀c G23D and F₀c L31F are unable to oligomerize. Wild-type F₀c (lane 1), F₀c G23D (lane 2), and F₀c L31F (lane 3) were synthesized in the presence of YidC proteoliposomes and analyzed by BN-PAGE as described under "Experimental Procedures."

what more pronounced with the F₀c L31F mutant, suggesting the presence of a very small Δ pH (Fig. 2C). When the oxidizable substrate NADH was added as an energy source all IMVs were capable of generating a Δ pH, showing that the expression of the F₀c mutants does not interfere with the integrity of the membrane (Fig. 2, D–G). These data demonstrate that the F₀c mutants G23D and L31F are defective in the formation of an active F₁F₀ ATP synthase complex.

F₀c G23D and L31F Insert in a YidC-dependent Manner into the Membrane but Are Unable to Form Oligomeric Complexes—YidC is required for the efficient insertion of wild-type F₀c into liposomes and IMVs (15) and in intact cells (15, 16). To test the membrane insertion requirement of the F₀c mutants, the proteins were synthesized in a cell-free transcription/translation system and assayed for membrane insertion into liposomes and YidC proteoliposomes using a protease protection assay (15). In the presence of YidC proteoliposomes, wild-type F₀c was protease resistant as shown before (15) (Fig. 3A, lane 2). F₀c G23D

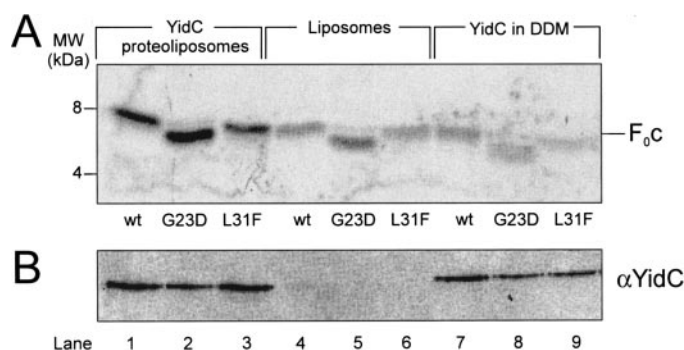


FIGURE 4. F₀c associates with YidC. A, wild-type (lanes 1, 4, 7), F₀c G23D (lanes 2, 5, 8), and F₀c L31F (lanes 3, 6, 9) proteins were synthesized in the presence of YidC proteoliposomes (lanes 1, 2, 3), liposomes (lanes 4, 5, 6), or without membranes (lanes 7, 8, 9). As a control for the binding activity in detergent solution, DDM-solubilized YidC proteoliposomes were added after the synthesis reaction was completed in the samples without membranes present (lanes 7, 8, 9). DDM was added to the samples to a concentration of 0.1%, and His-tagged YidC was subsequently purified with Ni²⁺-NTA beads. Co-affinity-purified F₀c was analyzed by SDS-PAGE and phosphorimaging. B, affinity-purified levels of YidC were determined by immunostaining.

and F₀c L31F were also found to be protease protected in the presence of YidC proteoliposomes (Fig. 3A, lanes 4 and 6), although insertion was less efficient as compared with wild-type F₀c. The formation of a second proteolytic fragment of F₀c L31F was observed at ~4 kDa (Fig. 3A, lane 8), which may point to an effect of the L31F mutation on the conformation of F₀c resulting in an altered susceptibility to the added protease. Similar results were obtained when IMVs were used instead of YidC proteoliposomes (data not shown). No or very little protease-protected F₀c could be detected in protein-free liposomes (Fig. 3B). These data demonstrate that the F₀c G23D and L31F mutants insert into the membrane in a YidC-dependent manner.

In vivo, the F₀c subunits assemble into a ring-like structure that forms part of the rotor domain of F₁F₀ ATP synthase (31–33). We have previously demonstrated that *in vitro*, YidC membrane-inserted wild-type F₀c assembles into an oligomer (15). To determine whether the membrane-inserted F₀c mutants are capable of oligomerization, YidC proteoliposomes containing membrane-inserted F₀c were re-isolated, solubilized with the detergent dodecyl maltoside, and subjected to Blue Native PAGE (Fig. 3C). Radioactively labeled wild-type F₀c was detected as a high molecular mass complex of ~80 kDa (Fig. 3C, lane 1) (15). The F₀c G23D and L31F mutants both failed to form such a complex and are only visible as a broad and a weak band, most likely the monomeric species (Fig. 3C, lanes 2 and 3). These data demonstrate that, at least *in vitro*, the YidC membrane-inserted F₀c G23D and L31F mutants are defective in the formation of a stable oligomer.

F₀c Associates with YidC in Vitro—Because the wild-type F₀c and assembly mutants require YidC for membrane insertion, the physical interaction between YidC and F₀c during membrane integration was addressed. Co-affinity purification was used to assay for the association of membrane-integrated YidC with *in vitro* synthesized and inserted radiolabeled F₀c. After an insertion reaction, membranes were solubilized with DDM. Unsolubilized membrane proteins were removed by centrifugation, and the supernatant was incubated with Ni²⁺-NTA

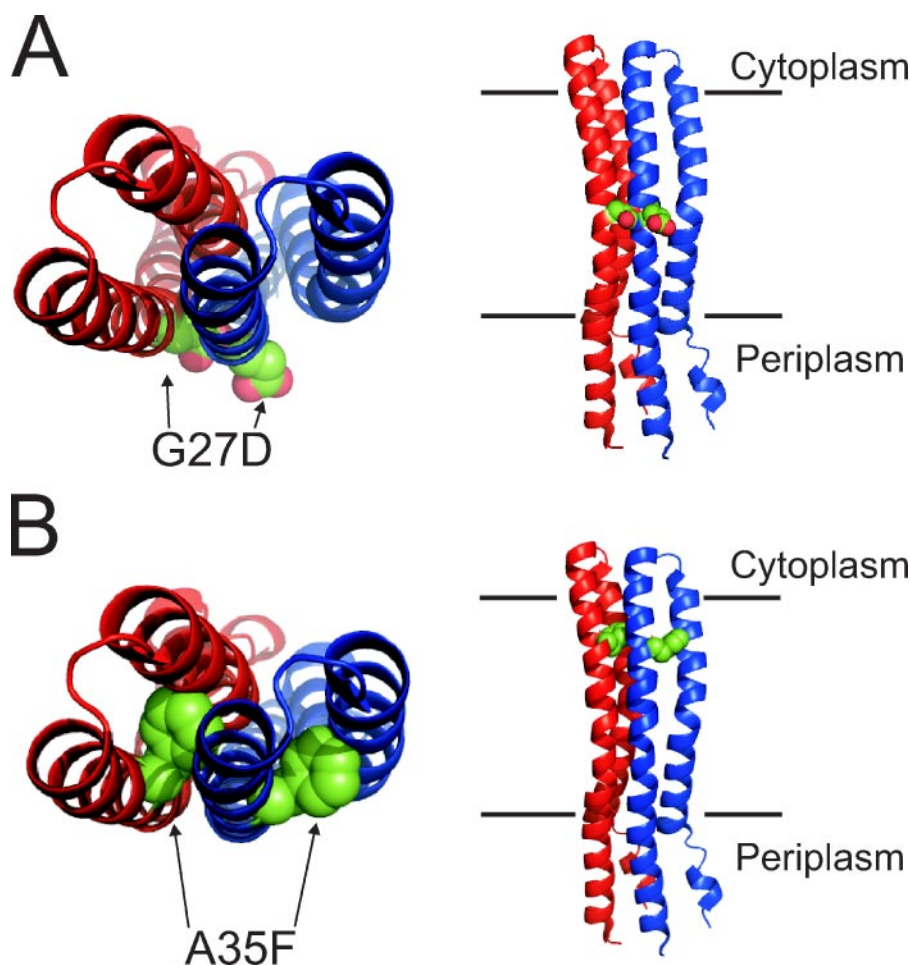


FIGURE 5. Structural model of the effect of the G23D and L31F mutations on the packing of two F_0c molecules (depicted in blue and red) based on the structure of the rotor ring of the F-type Na^+ -ATPase from *I. tartaricus* (Protein Data Bank code 1YCE). The side chains of the introduced aspartic acid and phenylalanine residues are shown as spheres with carbon atoms in green and oxygen atoms in red. Side and top (cytosolic face) views of F_0c G27D (A) and F_0c A35F (B) are shown. These images were created with PyMOL (pymol.sourceforge.net/).

beads to purify His-tagged YidC. Co-purification of F_0c was subsequently analyzed by autoradiography. Immunoblotting showed that the recovery of YidC in all samples was identical (Fig. 4B). Only a low level of background binding of F_0c to Ni^{2+} -NTA beads is observed with protein-free liposomes (Fig. 4A, lanes 4–6), but in the presence of YidC-containing proteoliposomes significant levels of *in vitro* synthesized F_0c co-purified (Fig. 4A, lanes 1–3). Remarkably, the F_0c G23D mutant reproducibly co-purifies to an up to 2-fold higher extent than wild-type and F_0c L31F. Strikingly, a much lower level of association was observed when solubilized YidC proteoliposomes were added after the *in vitro* synthesis reaction had been completed (Fig. 4A, lanes 7–9). These data demonstrate that YidC directly interacts with F_0c within the membrane and that the G23D mutation in F_0c stabilizes this interaction.

DISCUSSION

The functional assembly of energy-transducing membrane protein complexes such as the cytochrome *bo*₃ oxidase and the F_1F_0 ATP synthase in *E. coli* is strongly affected by the cellular depletion of YidC (34). Recent *in vivo* and *in vitro* studies dem-

onstrate that key subunits of these energy transducing complexes strictly require YidC for membrane insertion (8–10, 15, 16, 35, 36). Reconstitution studies demonstrate that YidC alone mediates the stable membrane insertion of subunit c (F_0c) of the F_0 sector of the F_1F_0 ATP synthase. Upon membrane insertion, a large oligomeric complex is formed with a molecular mass reminiscent of that of the F_0c ring (15). Here we have characterized two mutants of F_0c that are unable to form a functional F_1F_0 ATP synthase. Our data suggest that the assembly defect of the mutants is due to a deficiency in the F_0c ring formation, whereas the membrane insertion of these mutant proteins still strictly depends on YidC. This study therefore separates the proposed chaperone and insertase functions of YidC in the biogenesis of the F_0 sector. *In vivo*, the defect of the two F_0c mutants may be even further strengthened by a rapid degradation of the unassembled F_0c subunits by FtsH (37). Although we cannot exclude the possibility that YidC is also actively involved in the oligomerization of F_0c , this process appears not to be mechanistically linked to the initial insertion. YidC may facilitate the hydrophobic partitioning of F_0c into the membrane by providing an amphiphilic surface

(38), and subsequent oligomerization of correctly inserted F_0c units upon their release from YidC may be spontaneous events as suggested previously (39).

By co-affinity purification we have demonstrated that YidC directly interacts with its substrate F_0c . This interaction was found to be more pronounced with one of the assembly mutants, F_0c G23D, which suggests a slower release of F_0c G23D by YidC. Because this effect is not as dramatic as the oligomerization defect, assembly is most likely interrupted because of the inability of the mutant c subunits to interact (see below). Chen *et al.* (13) have previously shown by photo-cross-linking that YidC interacts with an arrested nascent chain of the Sec-independent phage protein, Pf3 coat. However, photo-cross-linking is not a suitable approach to study the interaction between YidC and F_0c , because the formation of a helical hairpin, which has been suggested to drive the membrane integration of F_0c (38, 40), can only occur when both termini of the polypeptide chain have been released from the ribosome. Our co-affinity-purified YidC- F_0c complex most likely represents an insertion and assembly intermediate of the catalytic cycle.

The *in vivo* and biochemical characterizations of the mutant F₁F₀ ATP synthase complexes demonstrate that the F₀c G23D and L31F mutants are unable to support the formation of an active F₀ sector. The crystal structure of the c-ring of an F-type Na⁺ ATP synthase of *Ilyobacter tartaricus* (33) now provides clues as to why these mutants are disturbed in oligomerization. An aspartate residue introduced at position 23 (*I. tartaricus* Gly-27) protrudes laterally from one F₀c subunit, resulting in both intra- and intermolecular steric hindrances (Fig. 5A), whereas the bulkier phenylalanine residue at position 31 (*I. tartaricus* Ala-35) causes a more extensive hindrance, but only within a single F₀c molecule (Fig. 5B). This difference in interactions might explain why the F₀c L31F mutant, in contrast to the F₀c G23D mutant, shows some ability to transport protons and, when overexpressed, rescues the growth defect on succinate. The intramolecular hindrances of the L31F mutant seem to destabilize the oligomer so that it does not survive native electrophoresis. However, oligomer formation is not completely abolished, because *in vivo*, partial activity of the F₁F₀ ATPase is retained. Moreover, the other F₀ subunits and the F₁ subcomplex may have a further stabilizing effect on the defective c-ring *in vivo*. The intermolecular hindrance of F₀c G23D disturbs the interactions between different subunits and consequently severely affects the assembly of the c-ring. It should be stressed that the level of F₀c in IMVs derived from cells expressing F₀c G23D is only marginally increased, which may point to degradation of incorrectly folded or conformationally unstable F₀c (37). The altered interaction between subunits, the partial membrane insertion defect and possibly a reduced transmembrane stability, and finally the slower release from YidC into the lipid bilayer will all contribute to a strongly reduced ability to form an active F₁F₀ ATPase complex, thus explaining the growth defect of F₀c G23D containing F₁F₀ ATPase on succinate.

The biogenesis of the active F₁F₀ ATPase is likely a coordinated process that requires the membrane insertion of the separate F₀ subunits, the stabilization of these subunits prior to their assembly into the F₀ subcomplex with the correct stoichiometry of a, b and c subunits, and finally the coupling of F₀ to the F₁ sector. It is not clear if the F₁-sector and the F₀-sector are fully assembled before coupling occurs, but *in vitro* studies demonstrate that mixing of the separately purified complexes results in the restoration of coupled ATPase activity (41). Previous studies have shown that F₀a is not present in the membrane unless F₀c and F₀b are co-expressed (42). *In vitro*, F₀a and F₀b can be isolated as a subcomplex that when co-reconstituted with F₀c yields a functional F₀ sector (43). F₀a is unstable when uncomplexed, and it is degraded by the FtsH protease (37). On the other hand, stable F₁F₀ complexes occur without F₀a present (44), and the activity of the subunit a-deprived complexes of the thermophilic *Bacillus* PS3 can be restored by the simple addition of purified F₀a (45). Fluorescence studies have shown that F₀b and a fully assembled c-ring suffice for the binding of the F₁ subcomplex (46). These data imply that a correct and timed assembly of the c-ring is crucial for the formation of a functional ATP synthase and that F₀a might be the last component that *in vivo* is added to a complex of F₀b and the c-ring.

The Alb3/Oxa1/YidC family of proteins are not only structurally but also functionally related (47, 48). In mitochondria, Oxa1p is involved in the membrane insertion of a subset of proteins synthesized in the mitochondrial matrix and interacts with translating ribosomes (49). Oxa1p is needed for the assembly of large energy-transducing complexes such as the F₁F₀ ATP synthase and the cytochrome c oxidase (50, 51). The thylakoidal Alb3 protein is required for the membrane insertion and assembly of light harvesting proteins (52). The results presented in this study do not exclude the possibility that YidC also actively participates in the assembly of large membrane complexes. In the case of F₀c, YidC might accumulate single or several membrane-inserted subunits prior to the formation of the ring structure. Our observation that stable YidC-F₀c subcomplexes can be isolated is consistent with this hypothesis. Future studies should address the mechanism by which the various subunits assemble into a functional F₀-sector and how protein substrates are released by YidC.

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